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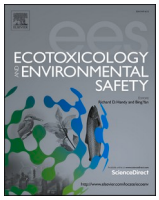

Development of a transcription-based bioanalytical tool to quantify the toxic potencies of hydrophilic compounds in water using the nematode *Caenorhabditis elegans*

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ABSTRACT

Low concentrations of environmental contaminants can be difficult to detect with current analytical tools, yet they may pose a risk to human and environmental health. The development of bioanalytical tools can help to quantify toxic potencies of biologically active compounds even of hydrophilic contaminants that are hard to extract from water samples. In this study, we exposed the model organism *Caenorhabditis elegans* synchronized in larval stage L4 to hydrophilic compounds via the water phase and analyzed the effect on gene transcription abundance. The nematodes were exposed to three direct-acting genotoxicants (1 mM and 5 mM): N-ethyl-N-nitrosourea (ENU), formaldehyde (HCHO), and methyl methanesulfonate (MMS). Genome-wide gene expression analysis using microarrays revealed significantly altered transcription levels of 495 genes for HCHO, 285 genes for ENU, and 569 genes for MMS in a concentration-dependent manner. A relatively high number of differentially expressed genes was downregulated, suggesting a general stress in nematodes treated with toxicants. Gene ontology and Kyoto encyclopedia of genes and genomes analysis demonstrated that the upregulated genes were primarily associated with metabolism, xenobiotic detoxification, proteotoxic stress, and innate immune response. Interestingly, genes downregulated by MMS were linked to the inhibition of neurotransmission, and this is in accordance with the observed decreased locomotion in MMS-exposed nematodes. Unexpectedly, the expression level of DNA damage response genes such as cell-cycle checkpoints or DNA-repair proteins were not altered. Overall, the current study shows that gene expression profiling of nematodes can be used to identify the potential mechanisms underlying the toxicity of chemical compounds. *C. elegans* is a promising test organism to further develop into a bioanalytical tool for quantification of the toxic potency of a wide array of hydrophilic contaminants.

1. Introduction

Chemical substances in the environment may pose a risk to human and environmental health. The contamination by pollutants with potential genotoxic and mutagenic effects has been previously documented in water sources (Ohe et al., 2004). Compounds may be present as parent molecules as well as their metabolites for which no analytical techniques exist yet or for which concentration is usually too low to detect chemically (Richardson et al., 2007). Hydrophilic pollutants are even more difficult to analyze in water because methods for extraction hardly exist (Stuart et al., 2012; Loos et al., 2013). This poses a problem, as testing the quality of drinking water sources typically involves the presence of hydrophilic compounds, of which especially genotoxic and endocrine disrupting compounds are of concern (Richardson et al., 2007; Gonsioroski et al., 2020). The development of chemical analytical tests for known individual agents will take many years and requires huge resources, still leaving questions on the total toxic potency of mixtures of these compounds such as mutagenicity or genotoxicity. In addition,

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biotransformation products may occur that we are not even aware of.

Living organisms, however, respond to bioactive compounds that they are exposed to. Biologically active contaminants undetectable by chemical analyses can still leave their signature in those organisms (Nuwaysir et al., 1999). This signature can be an alteration of gene expression patterns reflecting the mode of toxic action of the causative agent. In addition, transcriptional effects of chemical toxicants are not only mechanism-specific but could also be used to assess the toxic potency of complete mixtures (Gou et al., 2010; Poynton et al., 2008a).

Several developmental and toxicological studies have been conducted with the free-living soil nematode *Caenorhabditis elegans* as a model organism (Hunt, 2017; Leung et al., 2008). It provides particular experimental advantages such as small size, ease to handle, short life cycle, being invertebrate and relatively cheap to maintain in an ordinary laboratory setting. Most importantly, its genome has been completely sequenced and many genes or signaling pathways, particularly the ones involved in DNA damage response (DDR), are well conserved between *C. elegans* and higher organisms, hence comparable responses between the nematode and higher organisms are to be expected (Gartner et al., 2000; Hillier et al., 2005).

Several genes encoding DNA damage checkpoint proteins have been identified in *C. elegans* and are essential in sensing and responding to aberrations in their genetic material (Stergiou and Hengartner, 2004). The activation of checkpoints in response to DNA injuries typically stalls cell cycle progression to allow time for repair. If checkpoints fail to restore the DNA integrity, mutations can take place, and as response cell apoptosis occurs to prevent further problems (Ermolaeva and Schumacher, 2014). Other cellular responses that can be expected in response to genotoxic stress, include transcription regulating genes related to DNA repair, biotransformation enzymes, innate immune response and other mechanisms (Ermolaeva et al., 2013). Previous studies in *C. elegans* have primarily concentrated on ionizing radiation (Greiss et al., 2008; Schumacher et al., 2005), where pro-apoptotic genes such as *egl-1* and *ced-13* were transcriptionally induced in response to DNA damage. Although, several transcriptomics studies investigating transcriptional effects of other chemical agents have been carried out in the nematode (Cui et al., 2007; Viñuela et al., 2010), to our knowledge, this is the first genome-wide transcriptome study in *C. elegans* treated with genotoxic chemicals.

The afore described information has motivated us to use the transcriptional response of *C. elegans* for developing a small-scale in vivo bioassay as a biodetection and early warning system for the presence of genotoxic compounds. To develop such a bioanalytical tool, we chose N-ethyl-N-nitrosourea (ENU), formaldehyde (HCHO), and methyl methanesulfonate (MMS) as model compounds for determining genotoxic effects. These toxicants are known to directly react with nucleophilic sites (-NH, -OH and -SH) of macromolecules such as nucleic acids (i.e., DNA and RNA), enzymes, structural proteins, and other biological molecules (Beranek, 1990). It has been conclusively shown that ENU and MMS, which are monofunctional alkylating compounds, induce DNA injuries by reacting preferentially with ring nitrogen (N) and extra cyclic oxygen (O) atoms of nucleotides (Fu et al., 2012). Exposure to HCHO induces formation of crosslinks of DNA and proteins through electrophilic attacks ultimately leading to the impairment of normal cellular functions (Conaway et al., 1996).

The aim of this study was to develop an in vivo bioassay based on the genome-wide transcriptional response of *C. elegans* to direct-acting genotoxic compounds. Such an assay could simultaneously identify and quantify the toxic potency of single toxicants or mixtures. Gene expression profiling can provide insights in the type of toxic mechanisms involved and can be translated towards the nature of the toxicants present in a sample. Microarray analyses showed several genes in *C. elegans* whose expression level was differentially affected after 4 h exposure to the model genotoxicants. Surprisingly, no change was found in expression of most DDR genes, including the ones encoding for checkpoints and DNA repair proteins. The bioassay was validated by gene expression analyses using quantitative reverse transcription polymerase chain reaction (RT-qPCR) of selected gene targets from the microarray data.

2. Material and methods

2.1. Handling of nematode cultures

C. elegans wild-type strain (Bristol N2) nematodes were maintained on Nematode Growth Medium (NGM) agar plate at 16 °C (Stiernagle, 2006). Subsequently, nematode stocks were renewed every month using fresh NGM agar seeded with *E-coli* bacteria as source of food (Brenner, 1974). The experiments were conducted by using a nematode population of *C. elegans* N2 larvae (L4), grown synchronously at 20 °C for 48 h (starting from synchronized eggs) on a freshly prepared NGM agar plate seeded with *E. coli* strain OP50 to feed the nematodes. Synchronization was carried out by bleaching gravid nematodes with 5% sodium hypochlorite solution (Porta-de-la-Riva et al., 2012).

2.2. Chemical exposure

2.2.1. Exposure media

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were used without further purification. The stock solution of formaldehyde (HCHO) was prepared using the method of Moerman and Baillie (Moerman and Baillie, 1981). 61 mg of paraformaldehyde (Sigma-Aldrich P6148-500G) was warmed in 25 mL of 65 °C Milli-Q water and 0.1 M NaOH was added to clear the solution. The solution was diluted to 100 mL by adding M9 buffer, giving 20 mM solution adjusted to pH 7.04 with 0.1 M HCl. This solution was aliquoted in 1 mL, frozen, and stored at −20 °C. Methyl methane sulfonate (MMS, ≥ 99% purity) and N-ethyl-N-nitrosourea (ENU, 57% purity) were always freshly dissolved in M9 buffer to obtain 20 mM stock solutions which were further diluted to make the required concentrations. To obtain the required exposure concentration, stock solutions were further diluted in M9 buffer that was prepared according to Sulston et al. Sulston and Hodgkin (1988).

2.2.2. Exposure samples

A 4-hour exposure to the abovementioned toxicants were started in the synchronized L4 juvenile population. For microarray experiments, approximately 900 nematodes were exposed to two concentration (1 mM and 5 mM) and a control of M9 buffer in 1.5 mL Safe-Lock micro test tubes at 20 °C. Special care was taken to avoid temperature and developmental stage effects as these had shown to be relevant in the pilot experiment (Suppl. Pilot Study). After exposure, the nematodes were immediately pelleted by spinning the tubes in microcentrifuge for 20 s, 18,400 x g at room temperature, followed by removal of the supernatants. Subsequently, pellets were kept in the same exposure test tubes and flash-frozen in liquid nitrogen for 1 min before storing them at −80 °C until extraction of RNA. Three independent biological replicates were used per treatment in microarray experiments. For toxicity tests, duplicate samples were analyzed per treatment as described below.

2.3. Determination of non-lethal concentration

Non-lethal concentrations were determined for MMS, ENU, and HCHO to select the appropriate dose for microarray experiments. *C. elegans* L4-stage juveniles were exposed to three concentrations (1 mM, 5 mM, &10 mM), and a control of M9 buffer. For each exposure sample, 25–30 nematodes per independent test were divided in 4 wells of a 96-well tissue culture plate and treated in 135 μL of the exposure medium and control. After 4 h exposure, the nematodes were inspected under a stereo microscope to determine morbidity and mortality. The nematodes which failed to move in response to gentle probing by a platinum-wire based worm picker (Stiernagle, 2006) were counted as

dead. Two independent biological replicates were used per treatment.

2.4. Microarray gene expression analysis

2.4.1. RNA isolation

RNA was isolated following standard protocols as previously described (Jovic et al., 2017). In short, a Maxwell® 16 AS2000 instrument with a Maxwell® 16 LEV simplyRNA Tissue Kit (both Promega Corporation, Madison, WI, USA) was used following the manufacturer's protocol only modified at the lysis step. Each sample was treated with homogenization buffer (200 μL) and lysis buffer (200 μL), additionally, 10 μL of 20 mg/mL stock solution of proteinase K was added. Thereafter, the samples were incubated at 65 °C for 10 min to digest and remove proteins while shaking at 1000 rpm in a Thermomixer (Eppendorf, Hamburg, Germany). Subsequently, samples were cooled on ice and loaded into the cartridges provided by the manufacturer, thereby resuming the standard protocol.

2.4.2. Microarray preparation, hybridization, and scanning

Gene expression was measured using the Agilent *C. elegans* (V2) Gene Expression Microarray 4×44K slides following a procedure described before (Jovic et al., 2017). 'Two-Color Microarray-Based Gene Expression Analysis; Low Input Quick Amp Labeling'—protocol, version 6.0 from Agilent (Agilent Technologies, Santa Clara, CA, USA) was followed by cDNA synthesis, labelling with cy3 and cy5 dyes, and subsequent hybridization. Scanning was done using an Agilent High Resolution C Scanner with the settings as recommended by the protocol. For retrieval of the intensities the accompanying software was used (Agilent Feature Extract, v. 10.7.1.1). The array probe annotation was updated by blasting the probes against WS258 with blast (version 2.6.0, windows x64), using nblast with parameters: word size 7, reward 1, pentaly −3, and evalue 1. Probes with multiple hits were flagged and ignored in the analysis of affected genes. This microarray system holds 43,803 *C. elegans* probes. Detection is possible over a 5-log expression scale. In total, we could detect expression of 18,447 genes, representing > 90% of the nematode genome.

2.4.3. Normalization and pre-processing

Normalization of the data was done using the Limma package in "R" (version 3.4.2, x64) in RStudio (version 1.1.383). Arrays were normalized without background correction, normalization within arrays was done using the Loess method and between arrays using the quantile method (Smyth and Speed, 2003; Zahurak et al., 2007). The obtained values were $\log_2$ transformed and used for subsequent analysis. Initial analysis revealed the presence of batch- and dye-linked effects. Therefore, a batch correction was performed by fitting the gene expression to the linear model (Suppl. Eq. S1). After batch correction, a $\log_2$ ratio with the mean was calculated (Suppl. Eq. S2). For further analysis, the expression values of three biological replicates were averaged. The raw data of this experiment were submitted to ArrayExpress (E-MTAB-10265), accessible at https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-10264/.

2.4.4. Statistical data analysis

We did not employ intensity thresholds: we considered all data going into the analysis. Importantly, all models were using data taken from the level of the measurement (spots). Results were post-hoc translated to genes. To detect if the arrays were technically correct, we conducted a correlation analysis on the $\log_2$ ratio with the mean values using the *cor* function in "R". To identify the factors that can underlie variation in gene-expression, principal component analysis (PCA) was calculated on the $\log_2$ ratio with the mean values using the *prcomp* function in "R". To evaluate whether nematodes gene expression responded in a concentration-related manner a "*concentration-dependent linear model*" incorporating the compound as well as the exposure concentration (0, 1, and 5 mM) was applied to ENU, HCHO, and MMS separately (Suppl. Eq. S3). The resulting p-values from linear model were corrected for multiple testing using the *p.adjust* function with the Benjamini and Hochberg method (Benjamini and Hochberg, 1995). To assess the differentially expressed genes (DEG) per exposure condition, we took a high significance level of $-\log_{10}(p) > 4$ (i.e., $p < 0.0001$; FDR < 0.05). Custom written scripts for the microarray analysis are available at https://git.wur.nl/published_papers/karengera_2021_bioanalytical_tool_genotox.

2.4.5. Gene ontology (GO) and pathway enrichment analysis

All DEG lists generated by microarray experiments were uploaded to the "*Database for Annotation, Visualization and Integrated Discovery*" (DAVID) v6.8 (Huang et al., 2009) for KEGG pathway and for GO analyses in three categories, including biological processes (BP), molecular functions (MF), and cellular components (CC). For the enrichment analysis, settings were limited to Gene Ontology (GOTERM_BP_ALL, GOTERM_MF_ALL, and GOTERM_CC_ALL). A threshold False Discovery Rate (FDR) ≤0.05 was considered as strongly enriched in the annotation categories. The resulting GO terms were further used as input in the online software ReViGo (Supek et al., 2011) to summarize and remove the redundant terms. All default parameters were kept unchanged during the analysis.

2.5. Validation of 12 gene targets from microarray data by RT-qPCR

2.5.1. cDNA synthesis

RT-qPCR analyses were conducted on samples from control and nematodes exposed to 5 mM of toxicants. The RNA samples used in these experiments were from the same batches as used in the microarrays. The complementary DNA (cDNA) was synthesized from RNA template via reverse transcription (RT). The Invitrogen™ SuperScript™ IV VILO™ Master Mix with ezDNase™ Enzyme was used following the manufacturer's protocol. In short, 500 ng of total RNA was used as starting material in a 20 μL RT reaction. Each RT reaction involved two-minute digestion of genomic DNA (gDNA) using EZ DNase enzyme provided in the kit, followed by RT reaction in a T100™ Thermal Cycler. The annealing of primers was performed by incubation of samples at 25 °C for 10 min, reverse transcription at 50 °C for 10 min and inactivation of transcriptase enzyme at 85 °C for 5 min. The resulting cDNA was stored at −80 °C until further analysis.

2.5.2. PCR primer design and PCR reaction

Gene-specific PCR primers (115–200 bp) were designed by using three online database tools including Primer-BLAST (National Center for Biotechnology Information), Primer3 Input v. 0.4.0, and OligoAnalyzer v3.1 (Integrated DNA Technologies, Inc.). The specificity of primer pairs was initially checked in Primer-BLAST and confirmed by melting curve analysis. A temperature gradient qPCR (56–62 °C) was run to determine the optimal annealing temperature of each primer set. Prior to use in RT-qPCR, the cDNA stock obtained from a 500 ng RNA template was diluted 1:5 to match a 100-ng template as recommended by Invitrogen's user guide (Pub. No. MAN0015862 Rev. B.0. SuperScript™ IV VILO™ Master Mix). The 20 μL qPCR reaction mixtures were made of 6.8 μL PCR-grade (RNAse-free) water, 10 μL iQ™ SYBR® Green Supermix, 0.6 μL of specific forward and reverse primers (10 μM concentrated) and 2 μL of cDNA (5 ng/μL). Three independent biological replicate samples were analyzed per treatment and three technical replicates withing each sample were used. The cycling conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s, 62 °C for 30 s, and 72 °C for 30 s. Melting curve analysis was performed from 62 °C to 95 °C with an increment of 0.5 °C to confirm the amplification.

2.5.3. Validation of housekeeping genes for normalizing RNA expression

Eight candidate housekeeping genes were selected from our experiment and from the published studies (Hoogewijs et al., 2008). The

preliminary expression stability of these genes was further assessed in our microarray data. The expression levels of candidate housekeeping genes were measured by RT-qPCR method followed by ranking them according to expression stability, meaning that their levels were not influenced by the exposure. The selection of the most stable genes and the choice of the optimal number of housekeeping genes were computed by using geNorm algorithm according to Vandesompele et al. (2002) in "R" program v 3.5.2.

3. Results

3.1. Concentration-response relationship for lethal toxicity to C. elegans

To determine the non-toxic concentration range for studying the transcriptional effects of different genotoxic compounds, we first performed a concentration-response test (Fig. 1). The survival in all ENU exposed nematodes was not significantly different from control, and in the MMS-exposed nematodes only the 10 mM exposure resulted in significant (26%) reduced survival. Exposure to 5 and 10 mM HCHO resulted in significant dose-related mortality of respectively 39% and 70%.

The toxicity of the compounds was also qualitatively analyzed by comparing the swimming motions of the nematodes in the exposure media versus untreated worms. The worms exposed to 1 mM ENU and 1 mM MMS were actively swimming with a typical oscillatory movement (Park et al., 2008) similar to untreated nematodes. In contrast, most of the worms in 5 mM and 10 mM (ENU and MMS) or in 1 mM and 5 mM (HCHO) were motionless but slowly moved their bodies upon gentle touches with worm-picker probes. In 10 mM HCHO, most nematodes were lying with their bodies stretched motionless and barely moved upon probing. Based on the toxicity tests, we selected 5 mM as the maximum exposure concentration for microarray experiments.

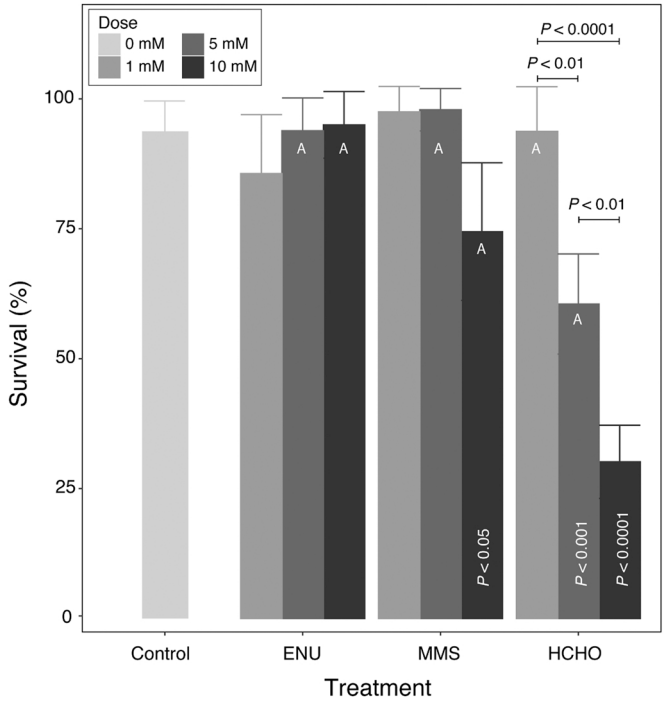

Fig. 1. Concentration-response relationship for lethal toxicity. Survival of *C. elegans* L4 larval stage after four hours exposure to 1 mM, 5 mM, or 10 mM of N-ethyl-N-nitrosourea (ENU), methyl methanesulfonate (MMS), and formaldehyde (HCHO). Plotted values are the means ± standard deviation (SD) for two independent biological replicates (n = 2) with about 25–30 nematodes divided in 4 wells per test. P-values (logistic regression) displayed vertically in chart bars indicate the significance of treatment compared to the untreated (control) samples while the horizontal Bonferroni-corrected P-values (logistic regression) (above the bars) show the significance of difference between concentrations within treatment. In groups indicated with A the nematodes displayed abnormal (reduced) swimming behavior.

3.2. Transcriptional response to genotoxic compounds

Pilot study (Suppl. Pilot Study) revealed the need to take extra caution on the experimental set-up to reduce potential batch development variation and temperature fluctuation effects. To determine the number of DEGs we used a concentration-dependent linear model. In general, there was a relatively high proportion of downregulated transcripts, where genes whose transcription was repressed counted 59% (for ENU), 46% (for HCHO), and 49% (for MMS) of the total DEGs in each treatment (Table 1). Relatively little overlap was found between gene transcripts affected by different treatments (Fig. 2). Only 7 genes (T20D4.12, C17C3.3, T07G12.5, K12C11.7, *ins-20*, C28G1.2, and D1086.2) overlapped between all three treatments and were all downregulated. These genes encode proteins involved in acyl-coA metabolic process (C17C3.3), transmembrane transport (T07G12.5), copper ion transmembrane transport (K12C11.7), and hormone activity (*ins-20*), whereas the function of T20D4.12, C28G1.2, and D1086.2 are not yet known.

To check whether these results were robust, a correlation analysis between the pilot study (2-hour exposure) and the main microarray experiment (4-hour exposure time) was conducted. We found a strong positive correlation for the array spots with the significance -log10(p) >4 (Fig. 3), meaning that despite differences in the exposure duration, similar transcriptional response trends were measured.

3.3. Functional analysis of differentially expressed genes (DEGs)

In general, a significant upregulation of genes related to metabolism and xenobiotics detoxification was found. For instance, concentration-dependent linear model analysis revealed that 57 out of 117 DEGs represented nearly half (49%) of the upregulated transcripts by ENU were enriched in metabolic processes, as annotated in the DAVID software (Fig. 4A and Suppl. Table S2). The affected genes include transcription factors belonging to nuclear hormone receptor family (*nhr-106, nhr-201, nhr-203, nhr-202*, and *nhr-237*) regulating gene expression, cytochrome P450 enzymes of phase 1 metabolism *(cyp-13A1, cyp-13A10, cyp-13A8, cyp-13A9, cyp-14A2*, and *cyp-33C8*), and phase 2 detoxification enzymes like glutathione-S-transferase enzymes *(gst-5, gst-6, gst-7, gst-8, gst-12, gst-13, gst-14, gst-31, gst-33,* and *gst-37*) or UDP-glucuronosyltransferase (*ugt-16, ugt-25, ugt-33,* and *ugt-44*). Metabolic processes were also induced by HCHO and MMS treatment (Suppl. Table S2), including the expression of *ugt-21, ugt-47, ugt-63, ugt-65*, and *cyp-29A2* genes by HCHO or *gst-30* by MMS. The results of GO analysis also revealed an upregulation of genes related to immune system process in the nematodes treated with ENU and MMS (Fig. 4A and Table 2). KEGG pathway enrichment analysis demonstrated that both HCHO and MMS induced the upregulation of genes involved in protein processing in endoplasmic reticulum (cel04141) (Suppl. Table S1). In addition, GO analysis in biological process (BP) category found that the induced transcripts by HCHO were related to protein folding (GO:0006457),

Table 1
The number of differentially expressed genes (DEGs) in *C. elegans* L4 following four-hour exposure to 1 mM and 5 mM N-ethyl-N-nitrosourea (ENU), formaldehyde (HCHO), and methyl methanesulfonate (MMS). Data were analyzed by a concentration-dependent linear model ($-\log_{10}(p) > 4$; FDR < 0.05).

	Upregulation	Downregulation	Total
ENU	117	168	285
HCHO	267	228	495
MMS	292	277	569

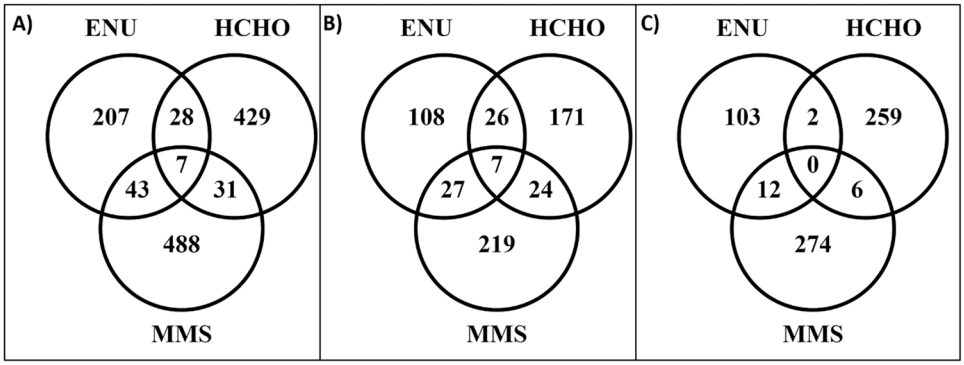

Fig. 2. Overlapping of differentially expressed genes (DEGs). Included are genes that responded to the treatments with N-ethyl-N-nitrosourea (ENU), formaldehyde (HCHO), and methyl methanesulfonate (MMS) in a concentration-dependent manner, as analysed by a linear model incorporating compound as well as exposure concentration. (A) the overlap between all differentially expressed genes, (B) the overlap between down-regulated genes, and (C) the overlap between up-regulated genes.

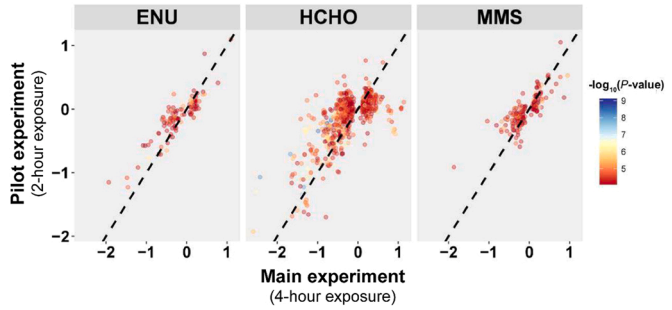

Fig. 3. Comparison of two independent microarray experiments. The outcome of the linear model of main experiment was compared to pilot (Supp. Pilot Study). Genes with a significant change in the expression above $-\log_{10}(p) = 4$ (displayed in the plot as color-coded dots based on their significance) showed a strong correlation (R = 0.89, R = 0.71, and R = 0.80, for N-ethyl-N-nitrosourea (ENU), formaldehyde (HCHO), and methyl methanesulfonate (MMS), respectively; $p < 1*10^{-38}$). These results show that despite the two-fold difference in the exposure duration, transcriptional effects of the toxic compounds are robust and replicable.

while the upregulated genes by MMS were associated with response to topologically incorrect protein (GO:0035966), response to endoplasmic reticulum stress (GO:0034976), and endoplasmic reticulum unfolded protein response (GO:0030968) (Suppl. Table S2).

A significant downregulation of genes involved in nerve impulse transmission was found in the nematodes treated with MMS (Fig. 4B and Table 2). These include genes like *mgl-1, dop-1, gab-1, gar-3, gar-1*, and *ser-4*, which were enriched in neuroactive ligand-receptor interaction pathway (cel04080). Treatment with HCHO reduced the expression levels of genes encoding proteins involved in the nematode defence responses during pathogen attacks from fungi or bacteria (e.g., *cnc-2, cnc-5, cnc-11, atg-16.2, lgg-1, nlp-29, sta-2, elt-7*, and *vhp-1*) (Fig. 4B and Table 2). Additional results of KEGG pathway and GO enrichment analysis are provided as supplementary information, including pathways (Suppl. Table S1), biological process (BP) terms (Suppl. Table S2), molecular function (MF) terms (Suppl. Table S3), and cellular component (CC) terms (Suppl. Table S4).

The individual annotation of all DEGs in DAVID software resulted in a list of genes that can be linked to the modes of action expected from the tested genotoxic model compounds such as transcriptional regulation, cell cycle regulation, proteotoxic stress, apoptosis, and other mechanisms (Suppl. Table S5). Nevertheless, KEGG pathway and GO analysis did not reveal any of the well-characterized DDR genes encoding for cell cycle checkpoints and DNA repair proteins to be transcriptionally

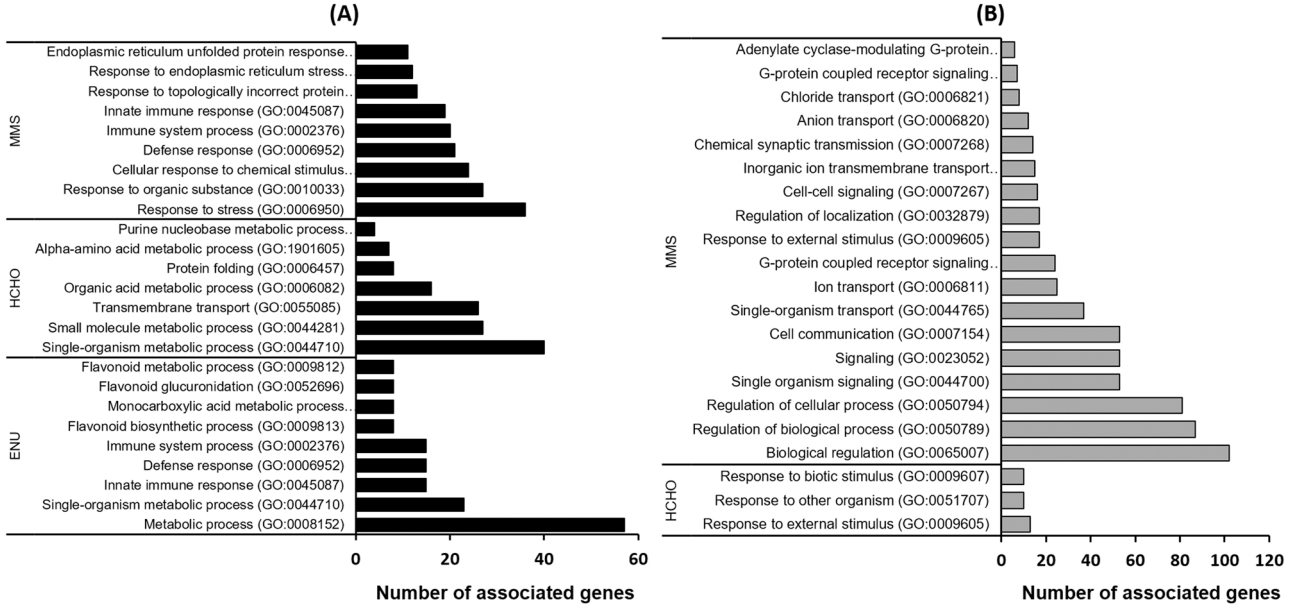

Fig. 4. Gene ontology enrichment analysis of differentially expressed genes in *C. elegans* L4. Plotted are gene ontologies in biological process category associated with upregulated genes (A) and with downregulated gene transcripts (B) following 4 h exposure to 1 mM and 5 mM N-ethyl-N-nitrosourea (ENU), formaldehyde (HCHO), and methyl methanesulfonate (MMS). The X-axis denotes number of genes significantly enriched in a gene ontology (GO) term (FDR < 0.05).

Table 2
Potential biological functions of genes down- or up-regulated in *C. elegans* L4 following 4 h exposure to 1 mM and 5 mM N-ethyl-N-nitrosourea (ENU), formaldehyde (HCHO), and methyl methanesulfonate (MMS). Differentially expressed genes (DEGs) are shown with their corresponding expression fold change (FC) values and the statistical significance expressed as $-\log_{10}(p)$.

Treatment	Gene	Protein	FC in 1 mM	FC in 5 mM	$-\log_{10}$ (p)
Defense response					
ENU	*atg-16.2*	Autophagic-related protein 16.2	-1.05	-1.24	5.57
HCHO	*atg-16.2*	Autophagic-related protein 16.2	-1.06	-1.40	4.07
HCHO	*cnc-11*	Caenacin (Caenorhabditis bacteriocin)	-1.74	-3.01	4.53
HCHO	*cnc-2*	Caenacin (Caenorhabditis bacteriocin)	-3.52	-19.70	6.06
HCHO	*cnc-5*	Caenacin (Caenorhabditis bacteriocin)	-1.29	-2.00	4.38
HCHO	*elt-7*	Transcription factor ELT-7	-1.10	-1.39	5.57
HCHO	*lgg-1*	Protein LGG-1	-1.05	-1.72	4.84
HCHO	*nlp-29*	QWGYGGY-amide	-2.84	-8.96	4.00
HCHO	*sta-2*	Signal transducer and activator of transcription b	-1.24	-2.28	4.38
HCHO	*vhp-1*	Tyrosine-protein phosphatase VHP-1	-1.03	-2.08	5.46
Signal transmission					
HCHO	*dop-1*	Dopamine receptor	-1.18	-1.45	4.09
MMS	*dop-1*	Dopamine receptor	-1.16	-1.53	6.11
MMS	*gab-1*	Gamma-aminobutyric acid receptor subunit beta	-1.05	-1.23	5.32
MMS	*gar-1*	Probable muscarinic acetylcholine receptor GAR-1	-1.03	-1.26	4.24
MMS	*gar-3*	Muscarinic acetylcholine receptor GAR-3	-1.15	-1.46	4.55
MMS	*mgl-1*	Metabotropic gLutamate receptor family	-1.07	-1.27	4.57
MMS	*ser-4*	SERotonin/octopamine receptor family	-1.13	-1.33	5.02
Innate immune response					
ENU	*cdr-4*	Cadmium responsive	1.43	3.48	5.47
MMS	*cdr-4*	Cadmium responsive	1.48	3.45	5.51
MMS	*fbxa-105*	F-box A protein	1.29	1.98	4.46
ENU	*fbxa-105*	F-box A protein	1.26	1.78	4.08
MMS	*gst-13*	Glutathione S-Transferase	1.33	2.25	6.33
ENU	*gst-13*	Glutathione S-Transferase	1.13	1.52	4.59
ENU	K08D8.4	hypothetical protein	1.24	2.51	5.07
MMS	K08D8.4	hypothetical protein	1.31	2.64	5.31
MMS	*lec-11*	Galectin	1.32	1.87	4.69
ENU	*lec-11*	Galectin	1.05	1.60	4.54
ENU	*ugt-44*	UDP-Glucuronosyltransferase	1.21	2.51	4.53
MMS	*ugt-44*	UDP-GlucuronosylTransferase	1.28	1.96	4.34
Oxidative stress response					
ENU	*gst-12*	Glutathione S-Transferase	1.57	3.55	5.04
MMS	*gst-12*	Glutathione S-Transferase	1.50	2.53	4.61
MMS	*gst-13*	Glutathione S-Transferase	1.33	2.25	6.33
ENU	*gst-13*	Glutathione S-Transferase	1.13	1.52	4.59
ENU	*gst-14*	Glutathione S-Transferase	1.45	2.80	4.74
ENU	*gst-31*	Glutathione S-Transferase	2.30	5.83	4.24
ENU	*gst-33*	Glutathione S-Transferase	1.49	3.68	6.68
ENU	*gst-37*	Glutathione S-Transferase	1.11	1.95	4.33
ENU	*gst-5*	Glutathione S-Transferase	1.56	4.43	7.39
ENU	*gst-6*	Probable glutathione S-transferase 6	1.20	1.60	4.83
ENU	*gst-7*	Probable glutathione S-transferase 7	1.14	1.78	4.59
ENU	*gst-8*	Probable glutathione S-transferase 8	1.18	1.66	5.01
Proteotoxic stress response					
MMS	*cul-6*	Cullin-6	1.14	2.14	4.45
MMS	*fbxa-158*	F-box A protein	1.43	3.36	7.48
MMS	*fbxa-75*	F-box A protein	1.64	6.81	6.58
MMS	*pals-22*	Protein containing ALS2cr12 (ALS2CR12) signature	1.15	1.46	5.37
MMS	*skr-3*	SKp1 Related (ubiquitin ligase complex component)	1.17	1.80	5.30
MMS	*skr-4*	SKp1 Related (ubiquitin ligase complex component)	1.10	1.58	5.87
Unfolded protein stress					
MMS	*arf-1.1*	ADP-ribosylation factor 1-like 1	1.17	2.18	5.21
MMS	C04F12.1	hypothetical protein	1.49	2.69	4.83
MMS	*ckb-2*	Choline kinase B2	2.30	6.74	6.08
MMS	*ckb-4*	Choline Kinase B	1.15	1.80	9.06
MMS	*cup-2*	Derlin-1	1.17	1.67	6.13
MMS	*dnj-7*	DNaJ domain (prokaryotic heat shock protein)	1.15	1.47	4.84
HCHO	*dnj-7*	DNaJ domain (prokaryotic heat shock protein)	1.10	1.36	4.70
MMS	*hsp-4*	Heat Shock Protein	1.34	2.53	6.69
MMS	*nsf-1*	Vesicle-fusing ATPase	1.05	1.35	5.41
HCHO	*pdi-2*	Protein disulfide isomerase	1.18	1.51	4.60
MMS	*pdi-2*	Protein disulfide isomerase	1.12	1.33	4.51
MMS	*rer-1*	Protein RER1 homolog	1.17	1.41	4.54
MMS	*sel-1*	Suppressor/Enhancer of LIN-12	1.03	1.27	5.34
MMS	Y54G2A.18	hypothetical protein	1.17	1.87	6.16

affected by any of the studied toxicants.

3.4. Validation of microarray data

To confirm the robustness of the results of microarray experiments, 12 gene targets selected from the main experiment were independently tested by using RT-qPCR experiment. RT-qPCR data were normalized by using four housekeeping genes including *csq-1, mdh-1, and pmp-3* selected from literature (Hoogewijs et al., 2008) and *ver-3* (from our study). The expression stability of these housekeeping genes in the exposed and unexposed nematodes was confirmed in our experimental conditions. RT-qPCR results showed similar expression trends to microarray outcomes (Suppl. Table S6).

4. Discussion

The nematode *C. elegans* has become an invaluable model organism in high-throughput screening assays to predict the toxicity of chemical substances (Cui et al., 2007; Viñuela et al., 2010). Despite this, very little was found in the literature on the whole-genome transcriptional effects of genotoxic compounds. In this work, we have successfully developed a bioassay based on exposure of L4 larval stage *C. elegans* as a test organism and using ENU, HCHO, and MMS as model genotoxicants. As shown by microarray results, we have established the optimal experimental conditions for generating optimal gene expression profiles of *C. elegans* in response to chemical exposure in liquid medium. Most importantly we showed that transcriptional effects were chemically specific and concentration dependent. In this assay we have also identified and validated 4 stable housekeeping genes that can be used to normalize gene expression quantitation in nematode by using RT-qPCR assay.

To our knowledge, no research has been carried out yet on genome-wide transcriptional responses of *C. elegans* to HCHO, ENU, and MMS or any comparable compounds that are known to directly induce DNA damage stress. Consequently, there was insufficient information in literature about the operating protocols including the exposure concentration for the testing of these substances in the nematodes. Therefore, we tested first the lethal toxicity of each compounds followed by the selection of the highest sublethal concentration to nematodes to maximize the occurrence of biological disturbances transcriptionally detectable. In addition, from our pilot microarray study we learned that subtle differences in the experimental temperature and the developmental synchronization of nematode culture could be major confounding variables that can influence gene expression profiling results.

The exposure concentrations used in this study caused a significant proportion of differentially expressed genes (DEGs) to be down-regulated. For HCHO-treated nematodes, transcriptional down-regulation was in line with the toxicity results, where the nematodes showed the highest mortality rate and impaired mobility, suggesting a predominance of general toxicity. A similar observation was previously reported in *Daphnia magna*, where high concentrations of copper, cadmium and zinc mostly trigged the transcriptional responses of general stress-related processes (Poynton et al., 2008b). Some genes among DEGs repressed by HCHO treatment have been previously characterized to have antibacterial and antifungal activity in *C. elegans* such as *nlp-29* gene (Lee et al., 2010). This suggests that nematodes affected by HCHO may be more vulnerable to infections.

The compounds tested in this study are known to react directly with biological molecules especially DNA and proteins via alkylation (Beranek, 1990). Consequently, upon exposure the nematodes were expected to initiate repair mechanisms in response to various molecular damages. In accordance with the proteotoxic behaviors of HCHO and MMS, there was indeed transcriptional evidence suggestive of protein damage stress, such as the upregulation of genes involved in the response to endoplasmic reticulum stress, unfolded proteins, or topologically incorrect protein. These results match those observed in other studies, in which several genes inducible by protein damage in *C. elegans* were identified for unfolded protein stress (*ckb-2, ckb-4*, C04F12.1, *pdi-2, dnj-7, cup-2, hsp-4, sel-1, arf-1.1, rer-1, nsf-1*, and Y54G2A.18) (Shen et al., 2005) and for proteotoxic stress response (*fbxa-75, fbxa-158, pals-22, skr-3, skr-4* and *cul-6*) (Panek et al., 2020).

While we anticipated that MMS and ENU would induce comparable transcriptional effects due to their close mode of toxicity as alkylating agents, ENU treatment did not appear to induce a significant expression among genes involved in proteotoxic stress response. Instead, expression of genes related to innate immune response, especially *cdr-4, fbxa-105, gst-13, lec-11, ugt-44*, and K08D8.4 was increased by both ENU and MMS treatments. In addition, the results of this study correlated with the mode of toxicity of ENU known to induce oxidative stress, as revealed by the transcriptional upregulation of peroxisome pathway (cel04146) and notably glutathione S-transferase (GST) gene family, such as *gst-5, gst-6, gst-7, gst-8, gst-12, gst-13, gst-14, gst-31, gst-33*, and *gst-37*, which have been previously linked to the oxidative stress resistance in *C. elegans* in response to exposure with chemicals like paraquat and juglone (Dues et al., 2017).

Our finding showed that MMS induced transcriptional repression of genes involved in nerve impulse transmission along a neuron, including *mgl-1, dop-1, gab-1, gar-3, gar-1*, and *ser-4*. These results correlated with our toxicity tests where the nematodes treated with MMS showed signs of the reduced motility. This is in the line with earlier literature that found an important relationship between the affected genes and locomotion in nematodes, such as dopaminergic receptor *dop-1* (Sanyal et al., 2004), serotonin receptor *ser-4* (Gürel et al., 2012), or muscarinic receptor *gar-3* (Chan et al., 2013). One may speculate the inactivation of neurotransmitters or receptor proteins by MMS through alkylation reaction resulted in the impairment of motor activity.

Having discussed how the exposure conditions investigated in our study induced the general stress in the nematodes, this raises the question of whether specific toxicity mechanisms may be eclipsed by the general ones as speculated by Gou et al. (2010). Our study suggests that the nematode might attempt to shut down parts of its gene-expression machinery to alleviate the general toxicity. In doing so, many biological processes may be affected, including processes related to the specific mode of action of the compound. Indeed, this could explain why we did not find significant changes in the transcription of genes that play a critical role in the maintenance of DNA integrity in *C. elegans* such as DNA-damage checkpoints or DNA repair proteins. Similar findings were reported upon exposure to X-ray radiation as this did not affect mRNA expression levels of DNA repair genes (Greiss et al., 2008), leading to the hypothesis that DDR genes in *C. elegans* might be instead regulated through posttranscriptional modifications of checkpoint proteins. Alternatively, the increase in metabolism and detoxification processes in the nematodes may have substantially reduced the efficacy of the tested toxicants, thus protecting the nematodes from genotoxic effects.

Despite the lack of expression change in DDR genes in our study, there was neither enough evidence to be able to conclude that DNA damage stress did not take place in the nematodes upon exposure to these compounds. Such DNA damage was demonstrated in a recent study showing that MMS-exposure of *C. elegans* generated a high number of mutations via base methylations (Volkova et al., 2020), and chemical mutagenesis in the nematode with ENU and HCHO is also well known (Kutscher and Shaham, n.d.). Moreover, our findings showed a significant change in the expression levels of genes like *gei-17* and *cul-6*, which have been reported to be involved in DNA-damage response in *C. elegans* (Kim and Michael, 2008). They were categorised differently in our study based on alternative functional descriptions and therefore not identified by either KEGG pathway or GO analyses. In addition, several genes involved in apoptosis or transcriptional regulation were expressed in this study, and data from literature has linked these processes to genotoxic response (Schumacher et al., 2005). Similarly, the induction of innate immune response found in our assay is reported in literature to be also triggered upon DNA damage stress (Ermolaeva et al., 2013).

Another aim of this study was to identify candidate marker genes that can be used to detect target toxicants. Previous studies have shown the potential of gene expression analysis to specifically detect contaminants in environmental samples (Poynton et al., 2008a). Based on our experimental work, several genes were found as potential candidate biomarkers for the detection of the tested compounds. In brief, each candidate gene expression biomarker complies with three criteria as proposed by Gou et al. (2010), including: a) the genes with chemical-specific response, b) the genes whose expression was concentration-dependent, and c) the genes which are linked to a specific mode of action related to the toxicant.

Overall, our study implies that both general as well as specific toxicity mechanisms of the tested compounds were operational in the nematodes and can be detected transcriptionally. Hence, this study will serve as a base for developing transcriptional biomarkers for detecting a wide array of bioactive contaminants, including hydrophilic ones that are hard to detect chemically. To determine reliable biomarkers, more studies like this should be carried out on several model compounds, mixtures and toxicants that require metabolic bioactivation. Our study successfully demonstrated the capability of this nematode to respond by a specific mode of action making it suitable for detection of specific compounds. It also showed that very high exposure concentrations most likely induce general stress that can mask specific effects. Further studies should focus on lower exposure concentrations to enable quantification of key transcriptional events specific to the mode of activity of a target compound. The applicability of this bioassay can be further improved by conducting transcriptomic concentration-response analysis of model compounds to define the concentration range detectable by this method and relate this to concentrations expected in field situations. The bioassay is expected to be not only mechanism-specific but also to indicate the exposure to compounds at concentrations far below those inducing physiological responses and before chronic effects become detectable. Thereby it could complement single-compound bioassays like CALUX (Been et al., 2021) or ToxTracker assay (Hendriks et al., 2012).

Author contribution

Antoine Karengera, Albertinka J. Murk, and Inez J. T. Dinkla conceived the theoretical framework. Joost A. G. Riksen provided technical support on the microarray experiments, Mark G. Sterken and H. Pieter J. van Veelen performed statistical analyses on microarrays, RT-qPCR, and toxicity data. Antoine Karengera planned and carried out the experiments. Antoine Karengera wrote the manuscript with input from Mark G. Sterken and Inez J. T. Dinkla, and in consultation with Albertinka J. Murk, Cong Bao, H. Pieter J. van Veelen, Jan E. Kammenga.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2021.112923.

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